# THE ROYAL SOCIETY

# The gall of subordination: changes in gall bladder function associated with social stress

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Diverse physiological and behavioural mechanisms allow animals to effectively deal with stressors, but chronic activation of the stress axis can have severe consequences. We explored the effects of chronic social stress on agonistic behaviour and gall bladder function, a critical but widely neglected component of stress-induced gastrointestinal dysfunction. Prolonged cohabitation with dominant individuals elicited behavioural modifications and dramatically increased bile retention in subordinate convict cichlid fish (*Archocentrus nigrofasciatum*). The key predictor of gall bladder hypertrophy was social subordination rather than status-related differences in food intake or body size. Stress-induced inhibition of gall bladder emptying could affect energy assimilation such that subordinate animals would not be able to effectively convert energy-rich food into mass gain. These results parallel changes in gall bladder function preceding cholesterol gallstone formation in humans and other mammals. Thus, social stress may be an important diagnostic criterion in understanding pathologies associated with gall bladder dysfunction.

**Keywords:** aggression; social subordination; gall bladder dysfunction; chronic stress; bile

## 1. INTRODUCTION

The stress response incorporates a host of neuroendocrinological, physiological and behavioural changes that allow animals to cope effectively with environmental perturbations. However, chronic stimulation of the stress axis can have significant negative consequences (Sapolsky 1994). One physiological effect of stress is the inhibition of digestive function, a mechanism that allows animals to divert blood flow and energy reserves to organs essential for dealing with the stressor. Stress-induced alterations to digestive physiology that have garnered the most empirical attention include inhibition of gastrointestinal motility (Coskun *et al.* 1997; Lee & Sarna 1997; Plourde 1999; Taché *et al.* 1999) and the acceleration of colonic transit (Mönnikes *et al.* 1993). These effects are mediated by corticotropin-releasing factor (CRF), a neuropeptide at the apex of the neuroendocrine stress axis (Mönnikes *et al.* 1993; Taché *et al.* 1999). Despite the prevalence of ailments associated with biliary dysfunction (e.g. gallstones), stress-induced changes in gall bladder emptying have received little empirical consideration (Lenz *et al.* 1992, 1993). Furthermore, subordination stress plays an integral role in the lives of gregarious animals but has been widely neglected in studies of digestive physiology. In this study, we demonstrate the effects of chronic social subordination on the physiology of subordinate animals, emphasizing status-dependent differences in bile retention and resultant gall bladder enlargement.

Cichlid fishes are useful models for laboratory studies on social subordination because they quickly establish dominance relationships for access to and in defence of mating territories (Oliveira & Almada 1998; Leiser & Itzkowitz 1999). Subordinates incur substantial fitness costs, including the loss of reproductive and/or foraging opportunities (Grand & Grant 1994; Wisenden 1995), and chronic subordination constitutes a behaviourally relevant stressor in these fishes (Fox *et al.* 1997). The effects of social subordination on serum glucocorticoid levels, which can act on many downstream targets, is well established in cichlids (Fox *et al.* 1997) and other vertebrates (Sapolsky 1994; Blanchard *et al.* 1995; Creel 2001; Sloman *et al.* 2001; Abbott *et al.* 2003), but the direct phenotypic consequences of chronic stress axis activation are less clear. Thus, we focused on one potent downstream physiological effect of chronic subordination, namely downregulation of digestive function in convict cichlids (*Archocentrus nigrofasciatum*).

## 2. MATERIAL AND METHODS

Convict cichlids were obtained commercially from SunPet Ltd. In experiment I, individuals were removed from large holding tanks (590 l), sexed by observation of the genital papilla with methylene blue, measured (total body length, standard length and body depth in centimetres) and weighed (grams). The water temperature in the holding tanks and the experimental tanks was maintained at 27 °C and the photoperiod at 12 L : 12 D. The fish were then isolated (38 l tanks) for 24 h to minimize the effects of previous social experience in the holding tanks on subsequent behaviour. Two males that differed in body mass by, on average, 33.8% ± 0.04 s.e.m. were then paired to ensure that one fish, the larger of the two, would unambiguously win the ensuing contest (small individuals: 3.24 g ± 0.68 s.e.m., large individuals: 6.53 g ± 0.92 s.e.m.). Each fish was placed behind a clear partition on opposite ends of a 38 l experimental tank for a 15 min acclimatization period. Experimental tanks were equipped with two terracotta shelters, a gravel substrate at 6 cm depth, and an external basket filtration system. After

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acclimatization, the clear partitions were lifted and the interactions between the small and large fish were observed for 15 min twice daily for 5 days. The data were collected live from a video monitor to reduce any interference of the observer with the interacting fish. All aggressive (e.g. lateral display, approach, attack, bite, chase, mouthwrestling and circle biting) and submissive (e.g. avoid, retreat, retreat to shelter) behaviour exhibited by the small fish plus the total time spent either in the shelter or hiding out (e.g. near the substrate or hovering in the top corners of the tank) was recorded; consult Enquist *et al.* (1990) for detailed descriptions of these behaviours in cichlid fishes. The fish were fed once daily following the second observation session. On the final day of the experiment, the fish were killed with a lethal dose of tricane methanosulphonate (Sigma) and weighed to determine post-interaction body mass. Necropsies were performed and the mass of the gonads, liver, gall bladder and bile determined; bile mass was assessed by subtracting the mass of damp-dry gall bladder tissue from the mass of the intact gall bladder. As mismatched cichlid pairs (i.e. substantial differences in body mass) were used in each trial to generate unambiguous dominance relationships, and because positive allometric relationships occur between body mass and most anatomical characters (see § 3), standardization methods are essential. As in Talbot & Higgins (1982), we corrected bile mass for liver mass because the gall bladder and liver are tightly linked both anatomically and functionally (e.g. the gall bladder is embedded in the liver and receives bile, produced by hepatocytes within the liver, through the bile duct); correcting for post-interaction body mass yielded similar results.

Experiment II entailed the same protocol as described in the previous paragraph except that both the small (5.31 g ± 0.06 s.e.m.) and large fish (3.95 g ± 0.15 s.e.m.) were observed once daily for 10 min in random order for 4 days and fed to satiation twice daily (after the first and second daily observation session). Feeding both fish to satiation entailed the following steps:

(i) floating flake food was added to the aquarium and the dominant animal fed, usually excluding the subordinate individual;

(ii) food was provided to the dominant until it ceased feeding and returned to the refuge;

(iii) once the dominant returned to the shelter, additional flake food was added until the subordinate animal stopped feeding.

Necropsies of the fish in experiment II were conducted in the same manner as described for experiment I except that the gastrointestinal tract was also removed and gut content was scored as empty, moderate or full. Isolated control animals ($N = 10$) were housed for 4–5 days in aquariums with the same characteristics as those in experiments I and II except only one shelter was provided; the control fish were sacrificed and their anatomical characteristics analysed as described for experiment II.

### (a) Statistical analyses

In experiment I, changes in the behaviour of the subordinate animal with time were examined using a multivariate repeated-measures analysis with 'day' as the explanatory variable and aggression $min^{-1}$, submission $min^{-1}$, percentage time in the shelter, and percentage time hiding out as the dependent variables. The overall multivariate analysis was significant ($F_{16,116} = 1.9$, $p < 0.05$), allowing for an examination of the univariate analyses with Dunn–Sidak adjustments of the $p$-values to control for compounding type I error. A similar multivariate analysis was conducted for experiment II but with both 'day' and 'status' as independent variables (all data reported in § 3). All multivariate analyses employed Pillai's Trace test statistic. The time of day that the subjects were sacrificed had no effect on measures of bile retention (covariate in multivariate analyses; main effect of time and interactions with size and experiment effects: $F_{1,25} < 0.93$, $p > 0.34$). The data for all parametric statistical analyses presented in the text and the figure legends were normally distributed. $p$-Values that are not accompanied by a test statistic indicate the results of a *post hoc* Fisher's PLSD multiple comparison. Body mass variance in the isolated controls and experimental animals was homogeneous ($\chi_1^2 = 0.0002$, $p = 0.99$) therefore making control versus experimental comparisons related to body mass more robust. Wilcoxon signed-rank tests were conducted to examine status-dependent differences in gut content scores; in this analysis, empty guts were scored as 1, moderately full guts as 2 and full guts as 3. Statistical comparisons between experiments I and II are provided in the legend of figure 1.

## 3. RESULTS

In all experimental trials, small individuals were clearly subordinate. In experiment I, small subordinate fish exhibited significantly lower rates of aggressive behaviour on days 2 through to 5 than on day 1 ($F_{4,29} = 5.7$, $p < 0.01$), demonstrated the same low rates of submissive behaviour, and spent the same percentage of time in the shelter and hiding out across days (latter three variables: $F_{4,29} < 1.8$, $p > 0.16$). In experiment II, there were no significant changes in the behaviour of the dominant or subordinate fish with time ($F_{16,272} = 1.4$, $p = 0.17$) but significant status-dependent behavioural differences emerged ($F_{4,13} = 16.9$, $p < 0.0001$). Dominant animals spent significantly more time in the shelter, aggressively excluding subordinates from it, and had higher rates of aggressive behaviour than subordinates ($F_{1,16} > 15.9$, $p < 0.001$). Subordinates spent significantly more time hiding out and had higher rates of submissive behaviour than dominant subjects ($F_{1,16} > 13.9$, $p < 0.002$).

To assess whether physiological changes occur concurrently with behavioural modifications as a consequence of social stress, we examined changes in body mass and bile retention in the gall bladders of both dominant and subordinate animals. Social status had a dramatic effect on body mass in the experimental animals (figure 1). Dominant animals gained (one sample $t_6 = 3.7$, $p < 0.01$) and subordinates lost (one sample $t_6 = -6.0$, $p < 0.001$) significant mass during the trial period, suggesting that dominant individuals monopolized the food resource. Furthermore, both the quantity and the quality (colour) of bile were affected by exposure to chronic social subordination (figures 1 and 2). Subordinates retained significantly more bile in the gall bladder than dominant animals (figure 1). Bile colour in dominant individuals ranged from colourless to yellow-green whereas bile colour in subordinate individuals was most often dark green (figure 2).

To distinguish whether increased bile retention was due to the social stressor or to decreased food intake, we conducted a second experiment where both the dominant and subordinate subjects were fed to satiation twice daily. Dominant animals continued to gain significant mass during the trial period (one sample $t_8 = 4.6$, $p < 0.01$;

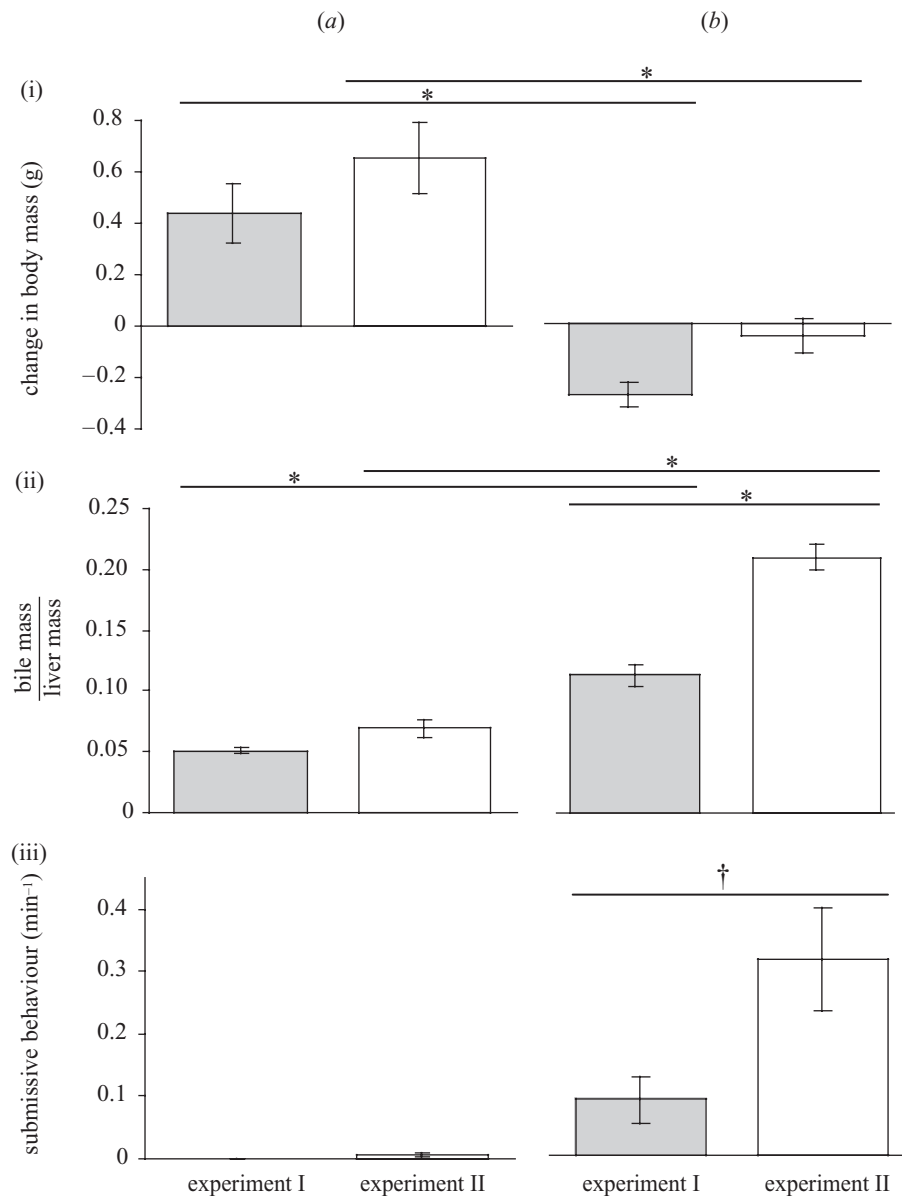Figure 1. Physiological and behavioural correlates of stress in (*a*) large, dominant and (*b*) small, subordinate convict cichlids. (i, ii) A comparison of mean change in body mass and mean bile mass over liver mass between dominant and subordinate animals and in experiment I (*n* = 7 contests; grey bars) versus experiment II (*n* = 9 contests; white bars). The overall multivariate analysis of variance indicated significant effects (experiment effect: $F_{2,27} = 23.9$, $p < 0.0001$, size effect: $F_{2,27} = 102.5$, $p < 0.0001$), thus independent univariate analyses with Dunn–Sidak adjustments of the *p*-values were conducted on bile mass over liver mass ($F_{3,28} = 79.4$, $p < 0.0001$) and change in body mass ($F_{3,28} = 17.1$, $p < 0.0001$). For each dependent variable, there were significant experiment ($F_{1,28} > 4.73$, $p < 0.038$) and status ($F_{1,28} > 46.5$, $p < 0.0001$) effects. Linear contrasts examined differences between dominant and subordinate animals and between subjects of similar status in experiment I versus experiment II. Lines above the histograms represent statistically significant contrasts (*$F_{1,28} > 21.3$, $p < 0.0001$). (iii) The mean number of submissive acts per minute of observation time in dominant and subordinate animals. An analysis of variance demonstrated significant differences in the rate of submissive behaviour exhibited by subordinate animals in experiment I versus experiment II (†$F_{1,14} = 5.7$, $p < 0.05$). Error bars represent ± 1 s.e.m.

figure 1) but, in this experiment, their subordinate counterparts did not lose significant mass (one sample $t_8 = -0.7$, $p = 0.49$; figure 1). The reduced loss of mass in subordinates, however, was not accompanied by a decrease in bile retention (figure 1). Subordinates in experiment II showed significantly more bile retention than those in experiment I (figure 1) and bile colour differences followed the same pattern as in experiment I (i.e. colourless to yellow-green in dominants; dark green in subordinates). Elevated bile retention coincided with a significant increase in the rate of submissive behaviour exhibited by subordinates

in experiment II relative to experiment I (figure 1). Regression analysis revealed a significant positive relationship between the rate of submissive behaviour and bile retention for subordinate animals in experiments I and II, implicating social subordination as a key predictor of gall bladder hypertrophy ($F_{1,14} = 8.9$, $p = 0.01$, $R^2 = 0.39$, $\beta = 0.15$; figure 3).

To further establish that the differences in bile retention among dominant and subordinate animals were not the result of differential feeding, we scored the gut contents of each subject in experiment II. There were no
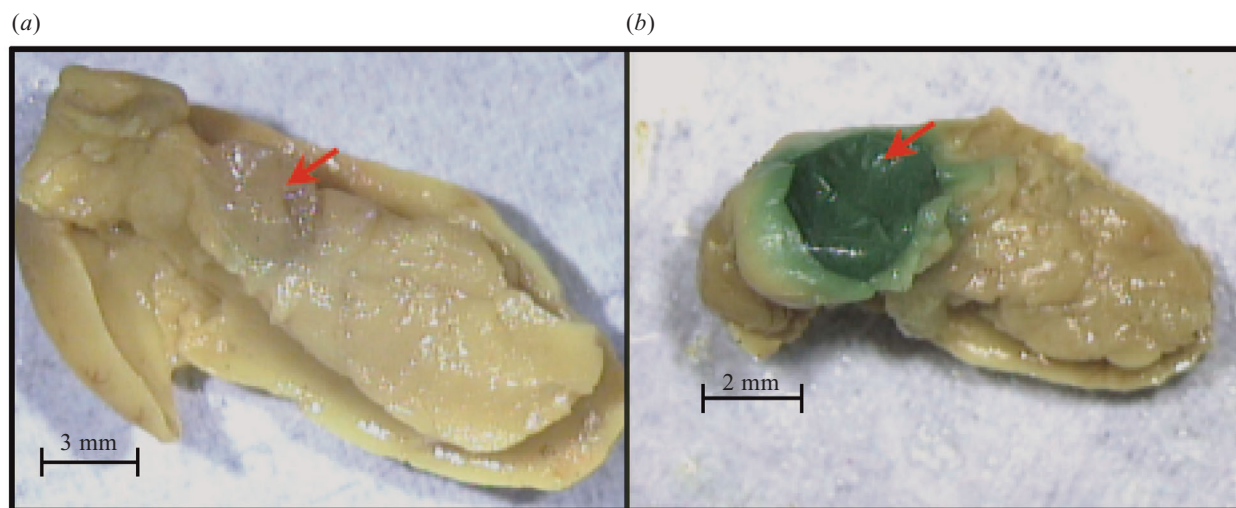
Figure 2. The size of the gall bladder relative to the liver and the coloration of the gall bladder (arrows) are noticeably different in (*a*) dominant versus (*b*) subordinate animals.
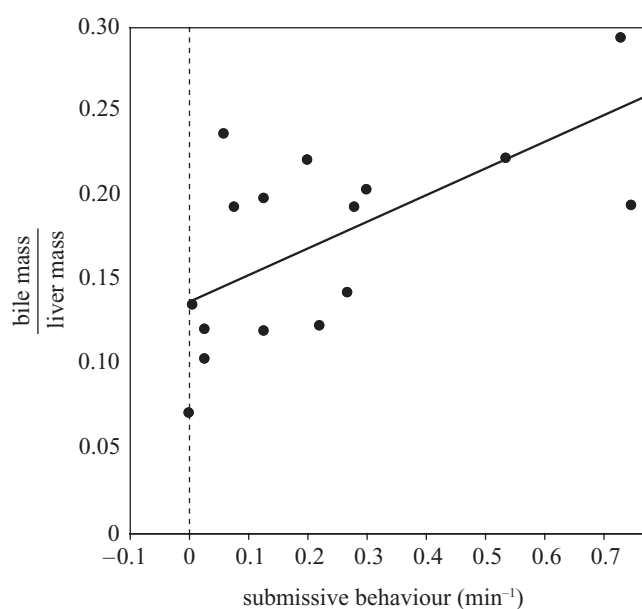


Figure 3. The relationship between the rate of submissive behaviour exhibited by the subordinates and corrected bile mass.

differences in gut content scores between dominant and subordinate animals (Wilcoxon signed rank: $Z = -1.0$, $p > 0.3$, $n = 9$ pairs) nor were there differences in mean bile mass across gut content categories (ANOVA; gut content: $F_{2,12} = 0.44$, $p = 0.66$, gut content $\times$ size: $F_{2,12} = 0.65$, $p = 0.54$). These results, and those described above, demonstrate that bile retention is influenced to a much greater extent by social subordination stress than by food intake, and that the degree of bile retention is closely related to the magnitude of the social stressor (i.e. rate of submissive behaviour).

We also examined whether bile retention was influenced more by differences in status than by differences in body mass. The relationship between body mass and bile mass was negatively allometric when both dominant and subordinate fish were included in the analysis (experiment I: $F_{1,12} = 65.9$, $p < 0.0001$, $R^2 = 0.85$; experiment II:

$F_{1,16} = 27.9$, $p < 0.0001$, $R^2 = 0.8$; both experiments: $F_{1,30} = 24.9$, $p < 0.001$, $R^2 = 0.45$). This negative relationship was in sharp contrast to all other anatomical characters measured including gonad, liver, gall bladder with bile, and uncorrected bile mass which showed significant positive allometry (all anatomical characters, both experiments: $F_{1,30} > 3.6$, $p < 0.06$, $0.33 < R^2 < 0.9$). To decouple the effects of size versus status, we conducted separate regression analyses for dominant and subordinate animals. The negative allometric relationship between body mass and bile mass was abolished when examined within a status class (dominant: $F_{1,14} = 0.57$, $p = 0.46$; subordinate: $F_{1,14} = 0.33$, $p = 0.57$). Thus, differences in bile retention between dominant and subordinate fish appear to be mediated by status asymmetries rather than size asymmetries.

Control animals gained significant mass while in isolation (mean $\pm$ s.e.m.: $0.426 \pm 0.03$; one sample $t_9 = 14.4$, $p < 0.0001$); mass increase in isolates was equivalent to that of the dominant fish ($p > 0.05$). Isolates retained significantly more bile (mean $\pm$ s.e.m.: $0.105 \pm 0.01$) than dominant fish in experiments I and II ($p < 0.01$), significantly less than subordinates in experiment II ($p < 0.0001$), and an equivalent amount to subordinates in experiment I ($p > 0.05$). There was no allometric relationship between body mass and bile mass in isolated animals ($F_{1,9} = 0.27$, $p = 0.62$). Food intake appeared to modulate bile flow in the absence of chronic social stress, an expected result if gall bladder emptying is normally meal- or cholecystokinin (CCK)-induced (Tortora & Grabowski 1996). Gut contents had a significant effect on bile retention in isolates (ANOVA: $F_{1,9} = 25.9$, $p < 0.001$); individuals whose gut fullness was scored as moderate (mean $\pm$ s.e.m.: $0.128 \pm 0.008$, $n = 6$) retained more bile than those whose guts were full (mean $\pm$ s.e.m.: $0.071 \pm 0.007$, $n = 4$). Social isolation has been described as a potent stressor in many species of fishes (Gómez-Laplaza & Morgan 2000). Isolates, however, exhibited intermediate changes in mass and bile retention when compared with the dominants and subordinates, suggesting that social isolation is a mild stressor relative to chronic social subordination.

## 4. DISCUSSION

We demonstrated that chronic subordination, a key stressor in the lives of social animals or those that aggregate during the reproductive season, drives dramatic changes in bile retention. These data are consistent with observations in other vertebrate taxa, including primates, showing that status-dependent physiological responses occur after the establishment of dominance relationships (Sapolsky 1994). At least two potential explanations exist for the differences in bile retention among dominant and subordinate individuals. First, stress resulting from chronic subordination could induce bile retention in subordinates. Alternatively, bile retention could be due to the inability of subordinates to obtain food; gall bladder contractions and bile flow are known to be meal induced and activated by CCK (Lenz *et al.* 1992; Mawe 1998), a hormone also involved in the inhibition of gastric emptying (Tortora & Grabowski 1996). Indeed, several studies on fishes have documented dramatic changes in gall bladder fullness and colour with feeding activity (e.g. starved animals retain more bile, which appears dark green rather than colourless or yellow; Talbot & Higgins 1982; McCormick & Podoliak 1984; Goede & Barton 1990). Nevertheless, comparisons between experiments I and II in this study clearly demonstrate that the degree of social subordination (i.e. rate of submissive behaviour) plays a critical role in bile retention, above and beyond any effects of differential feeding activity. However, the mechanism(s) by which social stress modulates gall bladder function is(are) unknown. Neurotransmitters, hormones, sex steroids and receptor-coupling proteins have all been implicated in the direct or indirect inhibition of gall bladder emptying (Gullo *et al.* 1986; Ryan 1987; Lenz *et al.* 1992, 1993; Mawe 1993; Tierney *et al.* 1994; Xiao *et al.* 1999; Guarraci *et al.* 2002). Furthermore, a subset of these molecules including CRF, norepinephrine and opioids have also been linked to social subordination stress (Huhman *et al.* 1992; Sgoifo *et al.* 1996; Albeck *et al.* 1997).

The limited data on mammals exposed to non-social stress (e.g. acoustic stress) or artificial activation of the stress axis (e.g. cerebral injection of CRF) indicate an indirect role for CRF in the inhibition of gall bladder emptying. CRF, an important trigger for both peripheral and central stress axis activity, may enhance bile retention through its stimulatory effect on norepinephrine and opioid production (Lenz *et al.* 1992, 1993; Mawe 1993, 1998; Guarraci *et al.* 2002). Norepinephrine and opioids inhibit CCK-induced gall bladder contraction presynaptically (e.g. binding to $\alpha_2$-adrenergic or opioid receptors, respectively) at the interface between vagal nerve terminals and gall bladder neurons or smooth muscle (Mawe 1993; Guarraci *et al.* 2002). Extended to our data on convict cichlids, these possible mechanisms imply a critical role for sympathetic modulation of gall bladder function in fishes exposed to chronic subordination. Indeed, this putative cascade of events leading to impairment of gall bladder function may be similar in taxa ranging from fishes to mammals, given the relatively conserved regulatory roles of both CRF and CCK (or CCK–gastrin-related peptides) across taxa (see Aldman & Holmgren 1995; Schjoldager *et al.* 1995; Nielsen *et al.* 1998; Seasholtz *et al.* 2002).

Our experiments also reveal the potential for an important relationship between social stress, gall bladder function, and energy assimilation. Subordinate animals either lost (experiment I) or failed to gain mass (experiment II), which could be because of the general catabolic effects associated with the stress response (Barton & Schreck 1987) or the synergistic effects of catabolism and a decreased ability to assimilate energy as a consequence of bile retention. In experiment II, subordinate animals were fed to satiation yet still exhibited dramatic increases in bile retention and minimal mass gain relative to dominant animals and controls. Thus, even under conditions where subordinate animals fed to satiation, stress-induced gall bladder dysfunction may impair energy assimilation (e.g. limited capacity to break down lipids and proteins) and restrict the allocation of food resources to other physiological processes (e.g. mass gain).

These data provide additional insights in terms of understanding issues related to the recently developed 'allostatic load hypothesis' (McEwen & Wingfield 2003). McEwen & Wingfield (2003) emphasize energy balance (i.e. available energy in the environment versus the energy required to maintain or restore normal physiological and behavioural function) as essential in regulating animals' responses to environmental challenges such as social subordination. They hypothesize that chronic stress may elicit prolonged alterations to or sustained activation of, for instance, the hypothalamic–pituitary–adrenal axis that can lead to pathologies even when food availability does not constrain the restoration of allostasis (i.e. type II allostatic overload). Our data suggest that chronic social stress could hinder assimilation of energy-rich foodstuffs by its effects on gall bladder emptying. Thus, pathologies may arise as a consequence of allostatic overload and concomitantly perpetuate imbalance over the long term by influencing the efficacy of energy acquisition. One of the most salient consequences of gall bladder dysfunction is the development of cholesterol gallstones and we argue that chronic social stress is likely to be a key environmental trigger for this condition.

Impairment of gall bladder contraction, the resultant retention of bile, and the potential for biochemical alterations to the bile (e.g. cholesterol supersaturation and crystallization, oxidation of bilirubin to biliverdin; McCormick & Podoliak 1984, fig. 2) in convict cichlids bear striking resemblance to the early stages of cholesterol gallstone formation in humans and other mammals (Ko & Lee 1999). Although post-operative and traumatic stressors have been linked to the onset of cholecystitis (Kaplan *et al.* 1983), the association between social stress and gallstone formation in humans, or any other vertebrate, remains elusive. In a comprehensive review of cholesterol gallstone formation, Apstein & Carey (1996) implicate cholesterol hypersecretion as the primary cause of gallstone formation but recognize impaired gall bladder emptying as an important secondary phenomenon.

The established link between CRF and the inhibition of CCK-induced gall bladder contraction indicates that social stress may promote gallstone formation through its action on secondary causative agents (e.g. arrest of gall bladder motility). Bile retention and gallstone formation can have debilitating consequences (Yoshidome *et al.* 2000; Cicala *et al.* 2001; Abou-Saif & Al-Kawas 2002)

and chronic stress probably enhances these risks. Indeed, recent studies have reported that the incidence of gallstone formation cannot be attributed solely to genetic factors and, thus, has a significant environmental component (Apstein & Carey 1996; Nakeeb *et al.* 2002). Our results provide an impetus for investigating chronic social stress (e.g. social phobias, anxiety disorders; Weinberg & Creed 2000; Wiedemann 2001; Condren *et al.* 2002) as a critical environmental factor influencing the prevalence of gallstone formation in human populations. In addition, our results implicate gall bladder function as an important diagnostic criterion in understanding the pathologies associated with social stress in a diverse array of vertebrate taxa, including humans.

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